



(11) **EP 2 223 971 B1**

(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention
of the grant of the patent:
09.11.2011 Bulletin 2011/45

(51) Int Cl.:
C09B 57/00 ^(2006.01) **C09B 23/10** ^(2006.01)
G01N 33/58 ^(2006.01)

(21) Application number: **10163379.0**

(22) Date of filing: **20.05.2005**

(54) **Long wavelength thiol-reactive fluorophores**

Langwellige Thiolgruppenreaktive Fluorophore

Fluoro-phones réactifs à thiol à onde longue

(84) Designated Contracting States:
**AT BE BG CH CY CZ DE DK EE ES FI FR GB GR
HU IE IS IT LI LT LU MC NL PL PT RO SE SI SK TR**

(30) Priority: **21.05.2004 US 573944 P**
06.08.2004 US 599514 P

(43) Date of publication of application:
01.09.2010 Bulletin 2010/35

(62) Document number(s) of the earlier application(s) in
accordance with Art. 76 EPC:
08017855.1 / 2 036 953
05812138.5 / 1 751 236

(73) Proprietor: **Becton, Dickinson & Company**
Franklin Lakes, NJ 07417-1880 (US)

(72) Inventors:
• **Pitner, Bruce, J.**
Durham, NC 27712 (US)
• **Sherman, Douglas, B.**
Durham, NC 27713 (US)
• **Ambroise, Arounaguiry**
Morrisville, NC 27560 (US)
• **Thomas, Joseph, K.**
Raleigh, NC 27613 (US)

(74) Representative: **von Kreisler Selting Werner**
Deichmannhaus am Dom
Bahnhofsvorplatz 1
50667 Köln (DE)

(56) References cited:
EP-A2- 0 383 092 EP-A2- 1 318 177

Note: Within nine months of the publication of the mention of the grant of the European patent in the European Patent Bulletin, any person may give notice to the European Patent Office of opposition to that patent, in accordance with the Implementing Regulations. Notice of opposition shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European Patent Convention).

EP 2 223 971 B1

Description**Field of the Invention**

[0001] The embodiments of the present invention are directed to long wavelength biosensors and their use in a method for the detection of an analyte.

Summary of the Embodiments

[0002] Fluorescent dyes or fluorophore compounds are suitable for use in various chemical and biological processes. Various embodiments are directed to fluorophores having a reactive group that can be used to couple or conjugate the fluorophore with another molecule such as a protein. Biosensors comprising fluorophores having a reactive group coupled or conjugated with a protein.

[0003] Fluorophores have a reactive group and have an emission wavelength of not less than 575 nm, referred to as near-infrared dyes (NIR dyes). In one embodiment, the fluorophores have an emission at 650 nm. The fluorophore embodiments include a pendant reactive group capable of conjugating with a member of a specific binding pair.

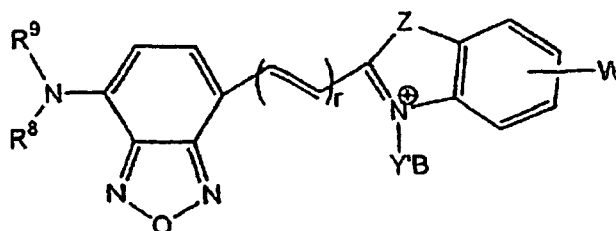
[0004] The fluorophores are suitable for coupling to receptors and to binding proteins having an affinity for a specific ligand or analyte. In various embodiments of the invention, the receptor or binding protein undergoes conformational changes when coupled to the ligand or analyte. The fluorophores when coupled to the binding protein exhibit a detectable signal change as a result of binding of ligand.

[0005] The fluorophore has a reactive moiety that can be covalently attached to an amino acid. The fluorophores in one embodiment have a thiol-reactive group that can be conjugated to a cysteine residue of a protein amino acid. Examples of suitable thiol-reactive groups that can be introduced into the fluorophore include a haloacetyl and particularly an iodoacetyl group. Other thiol-reactive groups include iodoacetamide, bromoacetamide, iodoacetate or maleimide.

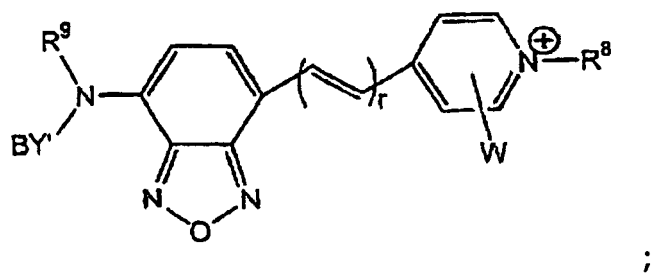
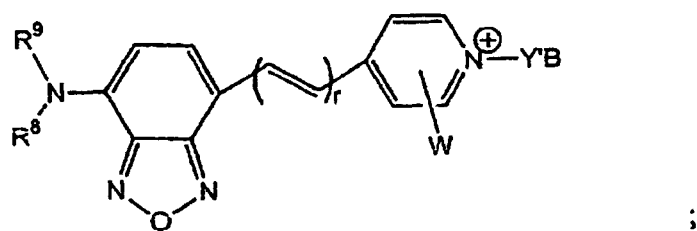
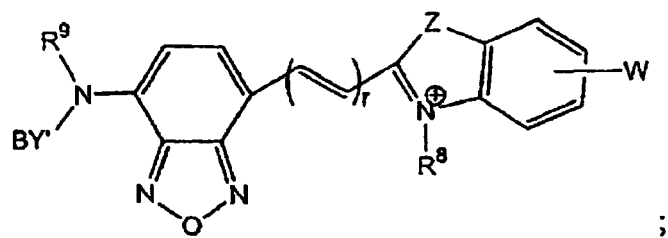
[0006] The fluorophore has a thiol-reactive group and has an emission of at least 575 nm. The fluorophores in the embodiment of the invention are benzoxadiazoles. In another embodiment, the invention is directed to derivatives of benzoxadiazole, hereinafter referred to interchangeably as benzoxadiazole nucleus or nuclei; or collectively as "fluorescent dye." Derivatives of the benzoxadiazole nuclei include any reaction product of the derivative, for example, with a protein amino acid group. Derivative is meant to include any chemical modification, addition, deletion, or substitution to an aforementioned nucleus. One embodiment includes nuclei of the aforementioned dyes that exhibit a fluorescence emission of at least 575 nm are included as embodiments. In one embodiment, the benzoxadiazole nuclei contains a thiol-reactive group for binding to a protein.

[0007] Another embodiment is also directed to a conjugate of a binding protein and a benzoxadiazole nucleus coupled to the binding protein through a cysteine residue on the binding protein. The cysteine residue of the protein can be naturally occurring or engineered into the protein. In one embodiment, the binding protein is a glucose binding protein that has an affinity for glucose and reversibly binds glucose. The fluorophore produces a detectable change in a fluorescence property in response to binding. The detectable change in a fluorescent property can be a shift in the wavelength of emission, a change in intensity of the emitted energy, a change in fluorescence lifetime, a change in anisotropy, change in polarization, or a combination thereof. In another embodiment, the binding protein is a maltose binding protein (MBP) that has an affinity for and binds maltose. In another embodiment, the binding protein is altered so that it has an affinity for and binds non-native ligands.

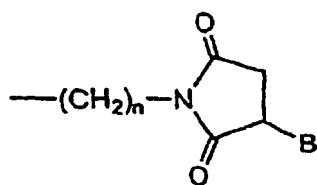
[0008] The various embodiments of the present invention provide for a biosensor compound selected from the group consisting of:



;

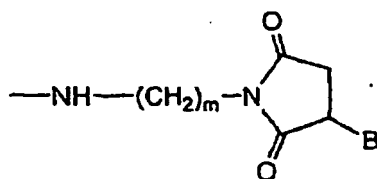


where Y'-B is



where n is an integer of 1 to 6,

or where Y'-B is A'-CO-V-B, where A' is -R²O- or -R²N(R³)-, where R² is a C₁ to C₆ alkyl, R³ is H or CH₃, and V-B is -CH₂-B or

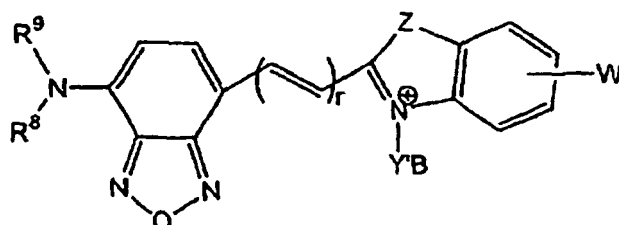


where m is an integer of 2 to 6,

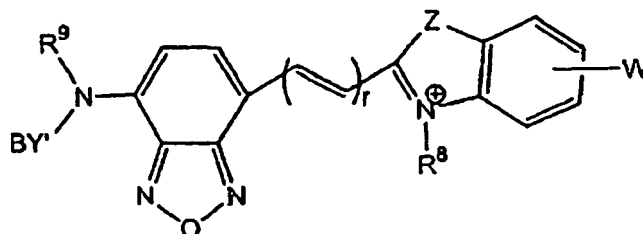
where r is an integer of 1 to 3, R^8 and R^9 are independently a C_1 to C_6 alkyl or $(CH_2)_sCO_2H$, where s is an integer of 2 to 5, Z is S, O, or $C(CH_3)_2$, W is H, CH_3 , SO_3H , fused benzene, or fused sulfobenzene, and

[0009] B is a receptor having a binding affinity for a ligand to be detected, and where said biosensor compound exhibits a detectable change in a fluorescence property as a result of changes in concentration of said ligand.

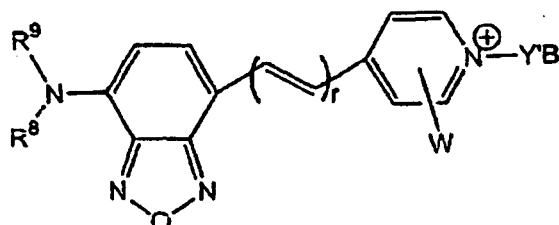
providing a biosensor compound having at least one mutated binding protein with a fluorophore covalently attached thereto through a thiol group of said binding protein, where said fluorophore exhibits an emission fluorescence of at least 575 nm and is selected from the group consisting of



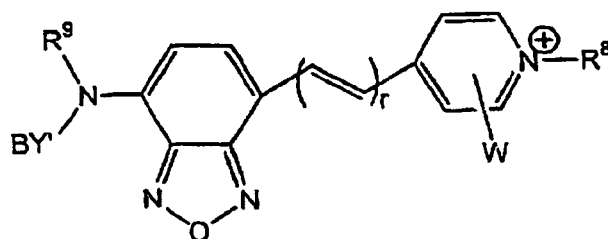
;



;

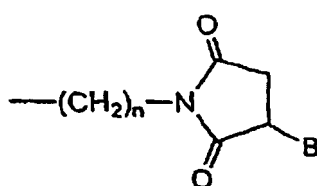


;



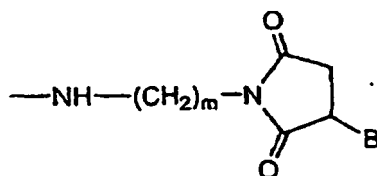
;

where Y'-B is



where n is an integer of 1 to 6,

or where Y'-B is A'-CO-V-B, where A' is -R²O- or -R²N(R³)-, where R² is a C₁ to C₆ alkyl, R³ is H or CH₃, and V-B is -CH₂-B or



where m is an integer of 2 to 6,

where r is an integer of 1 to 3, R⁸ and R⁹ are independently a C₁ to C₆ alkyl or (CH₂)₅CO₂H, where s is an integer of 2 to 5, Z is S, O, or C(CH₃)₂, W is H, CH₃, SO₃H fused benzene, or fused sulfobenzene, and

B is a receptor having a binding affinity for a ligand to be detected, and where said biosensor compound exhibits a detectable change in a fluorescence property as a result of changes in concentration of said ligand;

contacting said binding protein with an analyte source to bind said analyte to said binding protein; and

subjecting said binding protein to an energy source to excite said fluorophore and detecting a fluorescence property as an indicator of an analyte or analyte concentration in said analyte source.

[0010] Preferred embodiments of the invention are apparent from the dependent claims.

[0011] The biosensor compound is subjected to an energy source to excite said fluorophore and to detect a fluorescence property as an indicator of an analyte concentration in the analyte-containing source.

Detailed Description of Embodiments of the Invention

[0012] The present disclosure is directed to fluorescent dyes that are suitable for use as components of biosensors for detecting a ligand and particularly an analyte, and methods of use. One embodiment is directed to fluorescent dyes that can be conjugated to a receptor to detect, quantify, or detect and quantify the ligand.

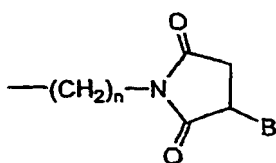
[0013] In one embodiment, the fluorescent dyes are used to produce a biosensor where the fluorescent dye is covalently attached to a binding protein. As used herein, the term "biosensor" and "biosensor compound" refers to a compound

that undergoes a detectable change in specific response to a ligand or target analyte. The embodiments of the biosensor discussed herein include a binding protein that is capable of binding to an analyte. In other embodiments, the biosensor of the invention is able to detect an analyte and to detect changes in the analyte concentration. In various embodiments, the protein may be chosen from the group of periplasmic binding proteins that includes, but is not limited to, glucose/galactose binding protein, maltose binding protein, allose-binding protein, arabinose-binding protein, dipeptide-binding protein, glutamic acid/aspartic acid-binding protein, glutamine binding protein, Fe(III)-binding protein, histidine-binding protein, leucine-binding protein, leucine/isoleucine/valine-binding protein, lysine/arginine/ornithine-binding protein, molybdate-binding protein, oligopeptide-binding protein, phosphate-binding protein, ribose-binding protein, sulfata-binding protein, Zn(II)-binding protein, and vitamin B-12-binding protein.

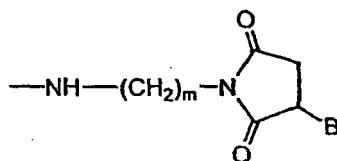
[0014] In other embodiments, the biosensor is a fluorescent dye covalently attached to a binding protein, wherein the protein-dye conjugate exhibits a fluorescence emission of 575 nm or higher. In one embodiment, the fluorescent dye exhibits a fluorescence emission of not less than 575 nm. In one exemplary form, the binding protein is a glucose/galactose binding protein (GGBP) that is able to bind with glucose when in contact with a glucose-containing source. In another embodiment, the binding protein is a maltose binding protein (MBP). Not to be held by any theory, the binding protein is understood to undergo a conformational change upon binding of ligand. The percentage of binding protein binding sites occupied by ligand is dependent upon the concentration of ligand and the binding constant of the binding protein.

[0015] The fluorescent dye embodiments that exhibit a fluorescence emission of at least about 575 nm avoid or minimize background interference from the biological system or other components in the glucose source. The fluorescent dyes exhibit a change in intensity of the fluorescence signal, a shift in the emission wavelength of the maximum fluorescence emission, a change in fluorescence lifetime, a change in anisotropy, a change in polarization, or a combination thereof, when the binding protein undergoes a conformational change as a result of changes in the glucose concentration. In the method embodiment, the biosensor contacts a sample containing analyte, for example glucose, to enable the analyte to bind with the binding protein, where the sample includes, but is not limited to, blood, saliva, interstitial fluid, etc. An energy source, such as a laser or LED, is applied to the biosensor to excite the fluorescent dye, and a fluorescence property is detected. Due to either a conformational change in the binding protein, subsequent changes in the microenvironment of the dye, or both, the detected fluorescence property or change of the detected fluorescence property can be correlated to the presence of an analyte or a analyte concentration. The fluorescence and detection can be carried out continuously or intermittently at predetermined times. Thus, episodic or continuous sensing of analyte, for example, glucose, is envisaged. The biosensor disclosed herein is adaptable for use in strips, implants, micro- and nano-particles, and the like.

[0016] The fluorescent dye is covalently attached to the binding protein in a site-specific manner to obtain the desired change in the fluorescence. The fluorescent dye is attached at a site on the binding protein so that the conformational Change maximizes the change in fluorescence properties. In other embodiments of the invention, the fluorescent dyes have a thiol-reactive group that can be coupled to the thiol group on a cysteine residue of the binding protein. The fluorescent dye includes the aforementioned derivatives of the benzoxadiazole nuclei. As noted above Y'B is



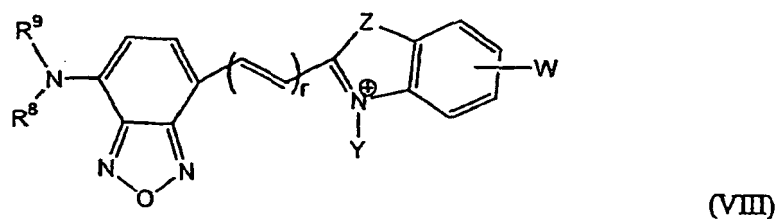
where n is an integer of 1 to 6, or Y'-B is A'-CO-V-B, where A' is -R²O- or -R²N(R³)-. R² is a C₁ to C₆ alkyl. In one embodiment, R² is a C₂ to C₄ alkyl. R³ is H or CH₃. V-B is -CH₂-B or



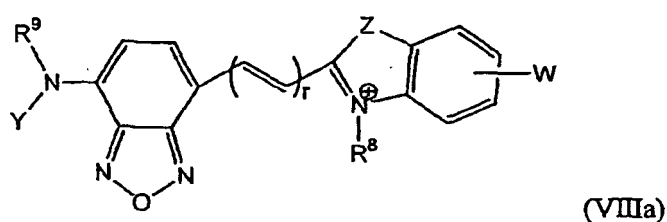
where m is an integer of 2 to 6. In one embodiment, R²O is -CH₂CH₂O-. In another embodiment, R²N(R³) is -CH₂CH₂NH-. The biosensor compound exhibits a detectable change in a fluorescence property as a result of changes in concentration

of the ligand. In one embodiment, B is a glucose/galactose binding protein that exhibits a detectable change in fluorescence emission as a result of changes in concentration of the ligand such as glucose. In another embodiment, B is a maltose binding protein.

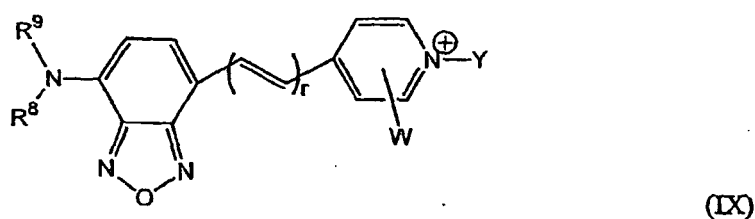
[0017] One embodiment includes benzoxadiazole nuclei having the formula



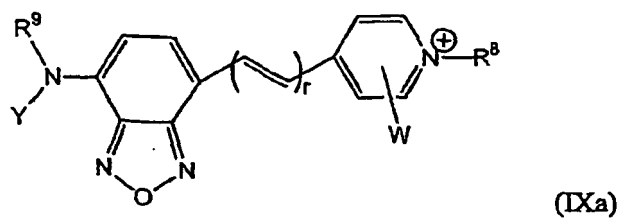
or



or

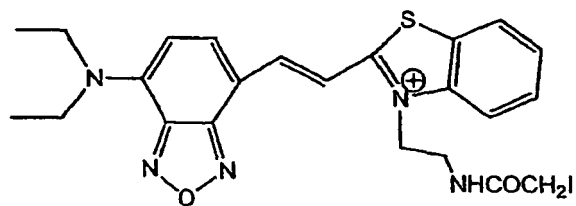


or

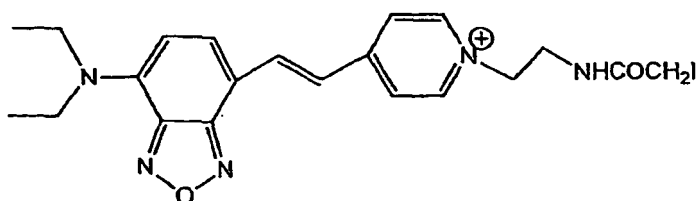


where r is an integer of 1 to 3, R^8 and R^9 are independently a C_1 to C_6 alkyl or $(CH_2)_sCO_2H$, where s is an integer of 2 to 5. Z is S, O, or $C(CH_3)_2$. W is H, CH_3 , SO_3H , fused benzene, or fused sulfobenzene. Y is as previously defined.

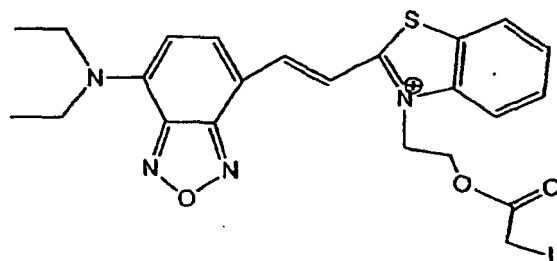
[0018] In one embodiment, the benzoxadiazole nucleus has the formula



(6)

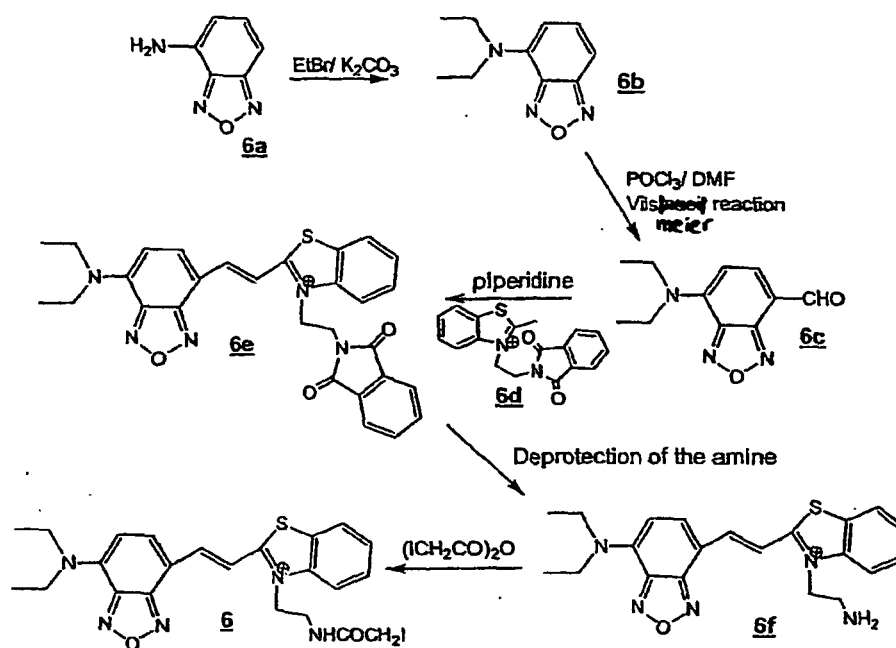


(7)



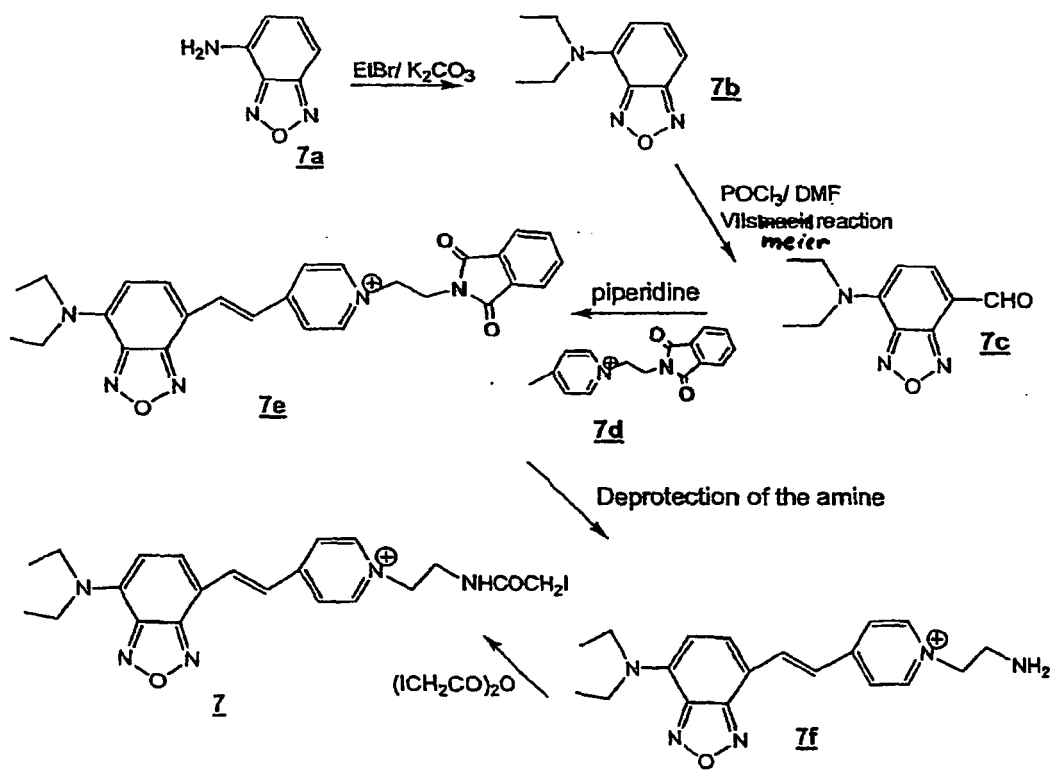
(8)

[0019] A procedure for producing benzoxadiazole nucleus derivative embodiments is depicted in the reaction Scheme V as follows.



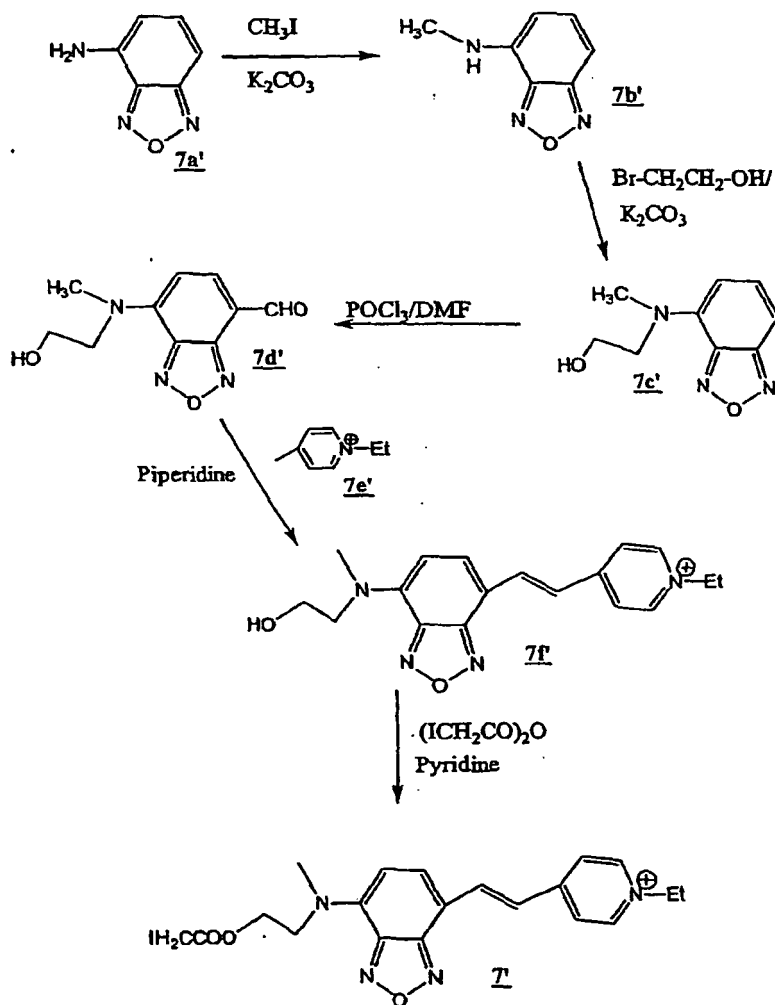
Scheme V

[0020] Scheme V can be modified to produce benzoxadiazole nucleus derivatives containing other ring systems, as shown below in Scheme VI.



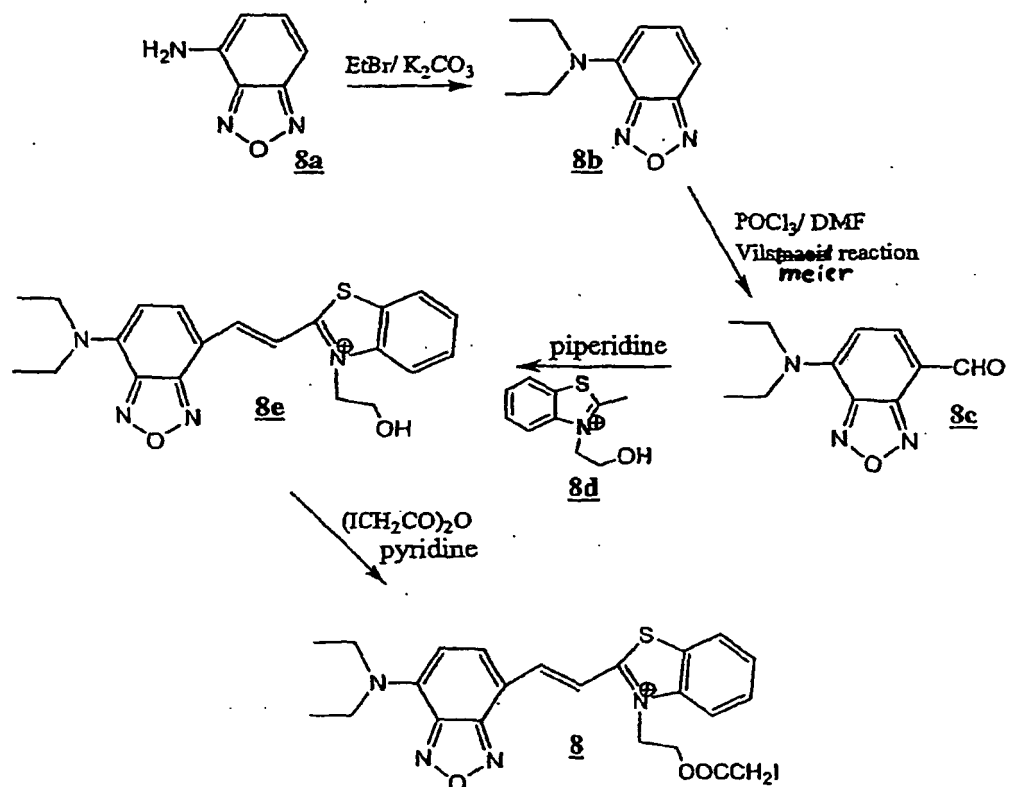
Scheme VI

[0021] An alternative procedure for producing benzoxadiazole nucleus derivatives is shown in reaction scheme VIa.



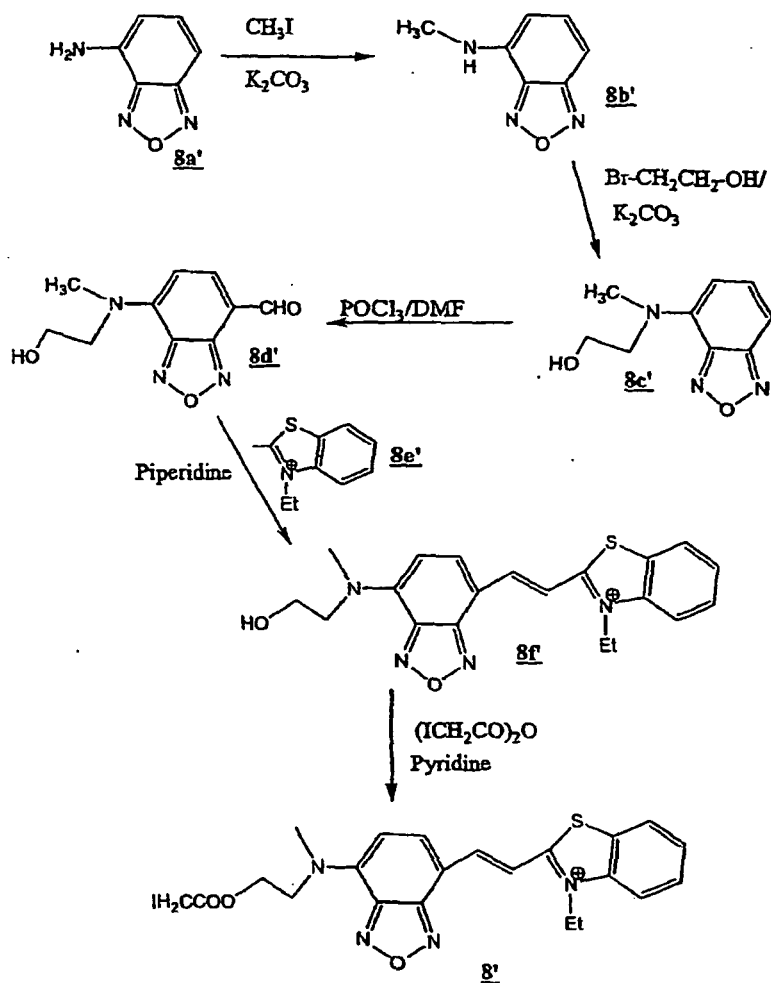
Scheme VIa

[0022] An exemplary procedure for the synthesis of benzoxadiazole nucleus with an iodoacetyl linker is depicted in Scheme VII



Scheme VII

[0023] An alternative procedure for producing benzoxadiazole nucleus derivatives is shown in the reaction Scheme VIIa

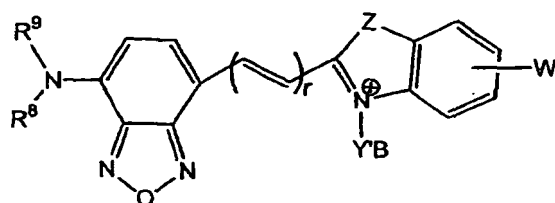


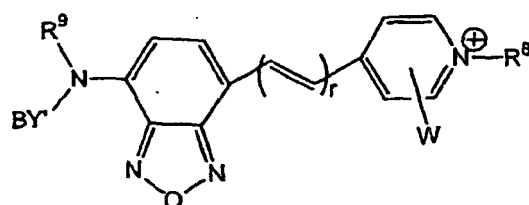
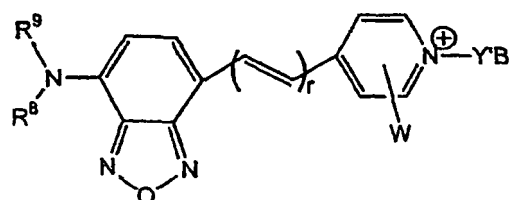
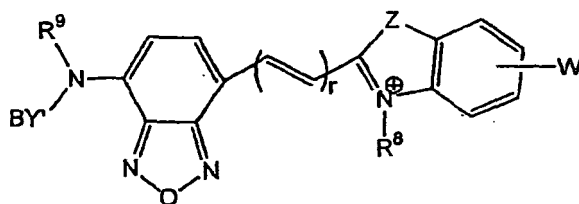
Scheme VIIa

[0024] In another embodiment the benzoxadiazole nucleus has a fluorescence emission. In one embodiment, the specific fluorescent nuclei described above have fluorescent emission above 575 nm.

[0025] In one embodiment, the resulting thiol-reactive nucleus is reacted with a binding protein to produce fluorescent binding protein conjugates useful as the biosensors of the invention

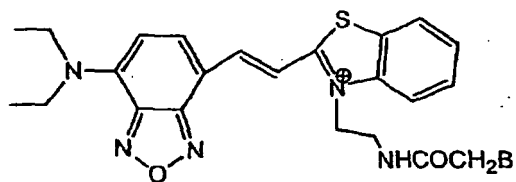
[0026] The resulting benzoxadiazole nucleus-binding protein conjugate has the Markush formulas:



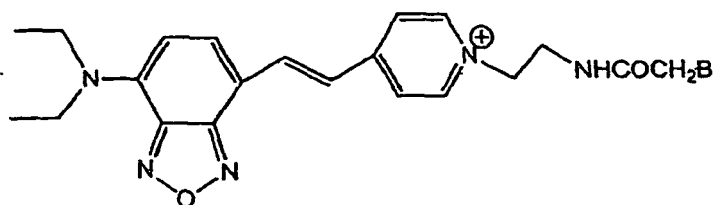


where Y'B is as previously defined and B is binding protein.

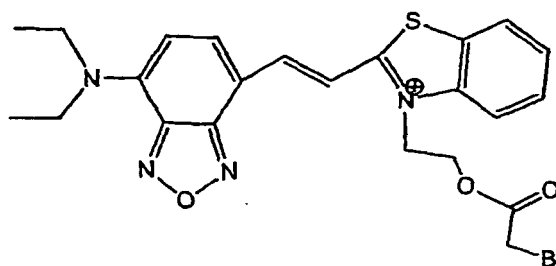
[0027] In one embodiment the resulting binding protein conjugate has the formula:



(XVII);



(XVIII)



(XIX)

[0028] The binding proteins B include a thiol group, for example, a cysteine residue, that is able to react with the thiol-reactive fluorescent dye. The term "binding proteins" refers to proteins that interact with specific analytes in a manner capable of transducing or providing a detectable signal differentiable either from when analyte is not present, analyte is present in varying concentrations over time, or in a concentration-dependent manner, by means of the methods described. Capable of transducing or "provide a detectable signal", as used herein, refers to the ability to recognize a change in a property of a reporter group in a manner that enables the detection of ligand-protein binding. For example, in one embodiment, the mutated GGBPs comprise a detectable reporter group whose detectable characteristics alter upon glucose binding. The change in the detectable characteristics may be due to an alteration in the environment of the label attached to the mutated GGBP or a conformational change of the protein resulting from binding. The transducing or providing a detectable signal may be reversible or non-reversible. As used herein, the terms transducing and providing a detectable signal are used interchangeably. The transduction event includes continuous, programmed, and episodic means, including one-time or reusable applications. Reversible signal transduction may be instantaneous or may be time-dependent, providing a correlation with the presence or concentration of analyte is established. Binding proteins mutated in such a manner to effect transduction are embodiments of the present invention. Binding proteins include, but are not limited to, glucose/galactose-binding protein, maltose-binding protein, alloose-binding protein, arabinose-binding protein, dipeptide-binding protein, glutamic acid/aspartic acid-binding protein, glutamine-binding protein, Fe (III)-binding protein, histidine-binding protein, leucine-binding protein, leucine/isoleudne/valine-binding protein, lysine/arginine/amithine-binding protein, molybdate-binding protein, oligopeptide-binding protein, phosphate-binding protein, nbose-binding protein, sulfate-binding protein, Zn(II)-binding protein, and vitamin B-12-binding protein.

[0029] The term "glucose/galactose binding protein" or "GGBP" or "maltose binding protein" or "MBP" as used herein refers to a type of protein naturally found in the periplasmic compartment of bacteria. These periplasmic proteins are naturally involved in chemotaxis and transport of small molecules (e.g., sugars, amino acids, and small peptides) into the cytoplasm. For example, GGBP is a single chain protein consisting of two globular α/β domains that are connected by three strands to form a hinge. The binding site is located in the cleft between the two domains. When glucose enters the binding site, GGBP undergoes a conformational change, centered at the hinge, which brings the two domains together and entraps glucose in the binding site. The wild type *E. coli* GGBP DNA and amino acid sequence can be found at www.ncbi.nlm.nih.gov/entrez/accession number D90885 (genomic clone) and accession number 23052 (amino acid sequence). In one embodiment, GGBP is from *E. coli*.

[0030] "Mutated binding protein" (for example "mutated GGBP") as used herein refers to binding proteins from bacteria wherein at least one amino acid has been substituted for, deleted from, or added to, the protein.

[0031] Mutations of binding proteins include for example, the addition or substitution of cysteine groups, non-naturally occurring amino acids, and replacement of substantially nonreactive amino acids with reactive amino acids.

[0032] Additional embodiments are mutations of the GGBP protein having a cysteine substituted for lysine at position 11 (K11C), a cysteine substituted for aspartic acid at position 14 (D14C), a cysteine substituted for valine at position 19 (V19C), a cysteine substituted for asparagine at position 43 (N43C), a cysteine substituted for glycine at position 74 (G74C), a cysteine substituted for tyrosine at position 107 (Y107C), a cysteine substituted for threonine at position 110 (T110C), a cysteine substituted for serine at position 112 (S 112C), a double mutant including a cysteine substituted for serine at position 112 and serine substituted for leucine at position 238 (S112C/L238S), a cysteine substituted for lysine at position 113 (K113C), a cysteine substituted for lysine at position 137 (K137C), a cysteine substituted for glutamic acid at position 149 (E149C), a double mutant including a cysteine substituted for glutamic acid at position 149 and an arginine substituted for alanine at position 213 (E149GA213R), a double mutant including a cysteine substituted for glutamic acid at position 149 and a serine substituted for leucine at position 238 (E149C/L238S), a double mutant including a serine substituted for alanine at position 213 and a cysteine substituted for histidine at position 152 (H152GA213S), a cysteine substituted for methionine at position 182 (M182C), a cysteine substituted for alanine at position 213 (A213C), a double mutant including a cysteine substituted for alanine at position 213 and a cysteine substituted for leucine at position 238 (A213C/L238C), a cysteine substituted for methionine at position 216 (M216C),

a cysteine substituted for aspartic acid at position 236 (D236C), a cysteine substituted for leucine at position 238 (L238C) a cysteine substituted for aspartic acid at position 287 (D287C), a cysteine substituted for arginine at position 292 (R292C), a cysteine substituted for valine at position 296 (V296C), a triple mutant including a cysteine substituted for glutamic acid at position 149 and a serine substituted for alanine at position 213 and a serine substituted for leucine at position 238 (B149C/A213S/L238S), a triple mutant including a cysteine substituted for glutamic acid at position 149 and an arginine substituted for alanine at position 213 and a serine substituted for leucine at position 238 (E149C/A213R/L238S), a cysteine substituted for glutamic acid at position 149 and a cysteine substituted for alanine at position 213 and a serine substituted for leucine at position 238 (E149C/A213C/L238S). Additional embodiments include mutations of GGBP at Y10C, N15C, Q26C, E93C, H152C, M182C, W183C, L255C, D257C, P294C, and V296C.

[0033] Additional embodiments are mutations of the maltose binding protein including, for example, D95C, F92C, I329C, S233C, and S337C.

[0034] Additional embodiments are histidine binding protein including, for example, E167C, K229C, V163C, Y230C, F231C, and Y88C.

[0035] Additional embodiments are mutations of the sulfate-binding protein including, for example, L65C, N70C, Q294C, R134C, W290C, and Y67C.

[0036] Additional embodiments are arabinose-binding protein including, for example, D275C, F23C, K301C, L253C, and L298C.

[0037] Additional embodiments are mutations of the dipeptide-binding protein including, for example, D450C, K394C, R141C, S111C, T44C, and W315C.

[0038] Additional embodiments are mutations of the glutamic acid/aspartic acid-binding protein including, for example, A207C, A210C, E119C, F126C, F131C, F270C, G211C, K268C, Q123C, and T129C.

[0039] Additional embodiments are mutations of the glutamine-binding protein including, for example, N160C, F221C, K219C, L162C, W220C, Y163C, and Y86C.

[0040] Additional embodiments are mutations of the Fe(III)-binding protein including, for example, E203C, K202C, K85C, and V287C.

[0041] Additional embodiments are mutations of the ribose-binding protein including, for example, T135C, D165C, E192C, A234C, L236C, and L265C.

[0042] Additional embodiments are mutations of the phosphate-binding protein including, for example, A225C, N223C, N226C, S164C, S39C, and A197C.

[0043] The mutation may serve one or more of several purposes. For example, a naturally occurring protein may be mutated in order to change the long-term stability of the protein, to conjugate the protein to a particular encapsulation matrix or polymer, to provide binding sites for detectable reporter groups, to adjust its binding constant with respect to a particular analyte, or combinations thereof. Long-term stability is intended to include thermal stability.

[0044] In one embodiment, analyte and mutated protein act as binding partners. The term "associates" or "binds" as used herein refers to binding partners having a relative binding constant (K_d) sufficiently strong to allow detection of binding to the protein by a detection means. The K_d may be calculated as the concentration of free analyte at which half the protein is bound, or vice versa. When the analyte of interest is glucose, the K_d values for the binding partners are between 0.0001 mM and 50 mM.

[0045] The fluorescent label can be attached to the mutated protein, for example a GGBP, by any conventional means known in the art. For example, the reporter group may be attached via amines or carboxyl residues on the protein. Exemplary embodiments include covalent coupling via thiol groups on cysteine residues of the mutated or native protein. For example, for mutated GGBP, cysteines may be located at position 10, at position 11, position 14, at position 15, position 19, at position 26, at position 43, at position 74, at position 92, at position 93, position 107, position 110, position 112, at position 113, at position 137, at position 149, at position 152, at position 154, at position 182, at position 183, at position 186, at position 211, at position 213, at position 216, at position 238, at position 240, at position 242, at position 255, at position 257, at position 287, at position 292, at position 294, and at position 296.

[0046] Any thiol-reactive group known in the art may be used for attaching reporter groups such as fluorophores to the cysteine in a natural or an engineered or mutated protein. For example, iodoacetamide, bromoacetamide, or maleimide are well known thiol-reactive moieties that may be used for this purpose.

[0047] Fluorophores that operate at long emission wavelengths (for example, 575 nm or greater) are embodiments when the molecular sensor is to be used *in vivo*, for example, incorporated into an implantable biosensor device (the skin being opaque below 575 nm). Conjugates containing these fluorophores, for example, attached at various cysteine mutants constructed in mutated GGBPs, can be screened to identify which ones result in the largest change in fluorescence upon glucose binding.

[0048] The following examples demonstrate the various embodiments of the invention

Example 1

[0049] This example produces iodoacetamido benzoxadiazole nucleus **6** according to Scheme V.

[0050] Intermediate **6b**: Amino benzoxadiazole **6a** (10 mmol) was reacted with ethyl bromide (50 mmol) in the presence of anhydrous potassium carbonate. The product was purified by column chromatography over silica gel using chloroform and methanol to afford the intermediate **6b** in 65% yield.

Mol. Wt calculated for $C_{10}H_{13}N_3O$ is 191 (M+), found 191 (M+1) (FAB)

[0051] Intermediate **6c**: $POCl_3$ (1 mL) was added to anhydrous DMF (4 mL) kept at $-5^\circ C$ in a round-bottomed flask with stirring. To this mixture was added intermediate **6b** (0.4 g), and stirring continued for 1 h. The reaction was quenched by adding the reaction mixture to ice water (100 mL), followed by neutralization with 1N KOH (pH adjusted to -9.0). The product was extracted with methylene chloride, and the organic phase was dried over sodium sulfate. The product was purified by column chromatography over silica gel using chloroform to afford 85% of the intermediate **6c**.

1H NMR ($CDCl_3$) δ ppm: 1.36 (t, CH_3 , 6H); 3.91 (q, CH_2 , 4H); 6.19 (d, CH, 1H); 7.82 (d, CH, 1H); 10.01 (s, CH, 1H).

Mol. Wt calculated for $C_{11}H_{13}N_3O_2$ is 219(M+), found 220 (M+1) (FAB)

[0052] Intermediate **6e**: Intermediate **6c** (350 mg) was reacted with intermediate **6d** (644 mg, prepared in the same manner as intermediate **2b**) in anhydrous methanol under reflux for 6 h in the presence of piperidine (50 mg) to form the parent dye **6e**. The crystals that separated upon cooling were collected by filtration and then were purified by flash column chromatography over silica gel using a mixture of methanol (5%) and chloroform.

1H NMR ($CDCl_3$, TMS) δ ppm: 1.42 (t, CH_3 , 6H); 3.20 (t, 2H); 4.0 (q, CH_2 , 4H); 4.40 (t, CH_2 , 2H); 6.50 (s, CH, 1H); 6.51 (s, CH, 1H); 7.5-8.7 (m, aromatic, 10H).

Mol. Wt calculated for $C_{29}H_{26}N_5O_3S$ is 524(M+), found 524(FAB)

[0053] The obtained dye **6e** showed a polar sensitivity as in Table 1 below:

Table 1

Solvent	Relative Fluorescence
Methylene Chloride	100
Acetonitrile	62
Ethanol	66
Methanol	50
Water	1

[0054] Such solvent polarity sensitivity of a dye is indicative of its environmental-sensitivity when attached to protein.

[0055] The parent intermediate benzoxadiazole nucleus **6e** can be deprotected using Na_2S and water to produce dye **6f** and subsequently reacted with iodoacetic anhydride to form the target dye **6**.

Example 2

[0056] This example produces iodoacetamido benzoxadiazole nucleus **7** according to Scheme VI. Intermediate **7c** was produced in the same manner as **6c** from Example 6.

[0057] Intermediate **7d**: 4-picoline (1g) was reacted with 2-bromoethyl phthalimide (2.5 g) by heating at $125^\circ C$ for 12h. The colorless solid formed was purified by repeated washing with chloroform to yield 3 g (86 %) of the compound **7d**.

1H NMR (CD_3OD) δ ppm: 2.67 (s, CH_3 , 3H); 4.30 (t, CH_2 , 2H); 4.88 (t, CH_2 , 2H); 7.81 (m, 4H); 7.93 (d, 2H); 8.93 (m 2H). ^{13}C NMR (CD_3OD) δ ppm: 22.12, 39.63, 60.69, 124.49, 130.00, 132.90, 135.76, 145.45, 162.06, 169.20.

[0058] Intermediate **7e**: Intermediate **7c** (220 mg) was reacted with intermediate **7d** (347 mg) in anhydrous methanol under reflux for 6 h in the presence of piperidine (50 mg) to form the parent dye **7e**. The crude product was subjected to column chromatography over silica gel using methanol and chloroform (1: 9) to obtain **7e**.

[0059] Compound **7**: Deprotection of the phthalimide in **7e** provides intermediate **7f**, which is then reacted with iodoacetic anhydride to produce the final product, compound **7**.

Example 2-1

[0060] This example can be used to produce the iodoacetyl benzoxadiazole nucleus **7'** according to reaction Scheme VIa.

[0061] Compound **7a'** is reacted with an equivalent amount of methyl iodide in presence of potassium carbonate and

a phase transfer catalyst to form intermediate **7b'**. A subsequent reaction of intermediate **7b'** with 2-bromoethanol produces intermediate **7c'**. Vilsmaier reaction on intermediate **7c'** produces intermediate **7d'**, and a reaction of **7d'** with **7e'** produces intermediate **7f**. A reaction of **7f** with iodoacetic anhydride will produce the final compound **7'**.

Example 3

[0062] This example produces the iodoacetyl benzoxadiazole nucleus **8** according to Scheme VII.

[0063] Intermediate **8b**: Amino benzoxadiazole **8a** (10 mmol) was reacted with ethyl bromide (50 mmol) in the presence of anhydrous potassium carbonate. The product was purified by column chromatography over silica gel using chloroform and methanol to afford the intermediate **8b** in 65% yield.

Mol. Wt calculated for $C_{10}H_{13}N_3O$ is 191 (M+), found 191 (M+1) (FAB)

[0064] Intermediate **8c**: $POCl_3$ (1 mL) was added to anhydrous DMF (4 mL) kept at $\sim 5^\circ C$ in a round-bottomed flask with stirring. To this mixture was added intermediate **8b** (0.4 g), and stirring continued for 1 h. The reaction was quenched by adding the reaction mixture to ice water (100 mL), and was followed by neutralization with 1N KOH (pH adjusted to -9.0). The product was extracted with methylene chloride, and the organic phase was dried over sodium sulfate. The product was purified by column chromatography over silica gel using chloroform to afford 85% of the intermediate **8c**. 1H NMR ($CDCl_3$) δ ppm: 1.36 (t, CH_3 , 6H); 3.91 (q, CH_2 , 4H); 6.19 (d, CH, 1H); 7.82 (d, CH, 1H); 10.01 (s, CH, 1H).

Mol. Wt calculated for $C_{11}H_{13}N_3O_2$ is 219 (M+), found 220 (M+1) (FAB-MS).

[0065] Intermediate **8d**: A mixture of 2-methylbenzothiazole (2.24 g, 15 mmol) and 2-bromoethanol (2.90 g, 23 mmol) was taken in 25 mL flask. The reaction mixture was heated at $120^\circ C$ for 24h. After 24h the reaction mixture was cooled to room temperature and chloroform (20 mL) was added and stirred for 4h at room temperature. The solid product was filtered, washed with chloroform and dried to give the desired product **8d** as light brown solid. 1H NMR (CD_3OD) δ ppm: 3.26 (s, 3H), 4.06 (t, 2H), 4.94 (t, 2H), 7.82 (t, 1H), 7.90 (t, 1H), 8.25 - 8.32 (m, 2H). ^{13}C NMR (CD_3OD) δ ppm: 16.8, 52.3, 59.1, 117.0; 124.2, 128.5, 129.4, 129.7, 141.6, 178.1.

[0066] Intermediate **8e**: Intermediate **8c** (55 mg) was reacted with intermediate **8d** (70 mg) in anhydrous methanol under reflux for 5 h in the presence of piperidine (100 mg) to form the parent dye **8e**. The crystals that separated upon cooling were collected by filtration and confirmed by NMR and mass spectroscopy. 1H NMR (CD_3OD) δ ppm: 1.38 (t, 6H), 3.13 (t, 2H), 4.03 (q, 4H), 4.13 (t, 2H), 6.51 (d, 1H), 6.53 (d, 1H), 7.67 (m, 1H), 7.78 (m, 1H), 7.89 (m, 1H), 8.03 (m, 1H), 8.14 (m, 1H), 8.20 (m, 1H). ^{13}C NMR (CD_3OD) δ ppm: 11.62, 44.54, 51.08, 59.18, 104.72, 108.01, 109.18, 115.96, 123.33, 127.47, 127.70, 129.09, 142.10, 143.40, 144.25, 144.97, 145.35, 149.04, 172.69.

Mol. Wt calculated for $C_{29}H_{26}N_5O_3S$ is 395 (M+), found 395 (FAB MS).

[0067] Compound **8**: The parent dye **8e** (20 mg) was reacted with iodoacetic anhydride (20 mg) by stirring at room temperature (3h) in anhydrous methylene chloride (5 mL) in presence of pyridine (50 mg). The obtained product dye **8** was purified by precipitation from hexane. Mol. Wt calculated for $C_{23}H_{24}IN_4O_3S^+$ is 563 (M+), found 563 (FAB)

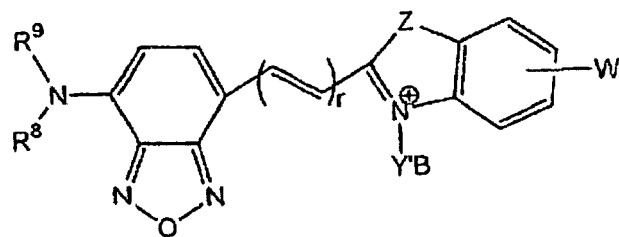
Example 3-1

[0068] This example produces the iodoacetyl benzoxadiazole nucleus **8'** according to reaction scheme VIIa.

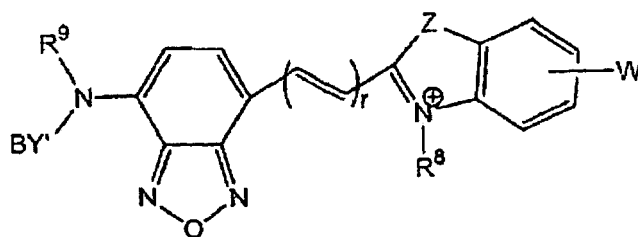
[0069] Compound **8a'** is reacted with an equivalent amount of methyl iodide in the presence of anhydrous potassium carbonate and a phase transfer catalyst to form the mono-methylamino derivative **8b'**. Compound **8b'** is then reacted with 2-bromoethanol in presence of potassium carbonate and phase transfer catalyst to form intermediate **8c'**. Vilsmeier reaction on **8c'** produces intermediate **8d'**, which is reacted with **8e'** to form intermediate **8f'**. A subsequent reaction of intermediate **8f'** with iodoacetic anhydride produces the final compound **8'**.

Claims

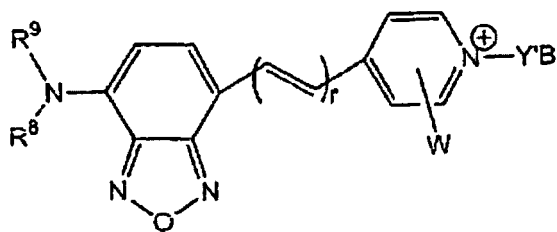
1. A biosensor compound selected from the group consisting of:



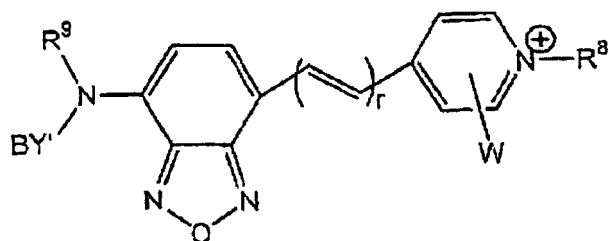
;



;

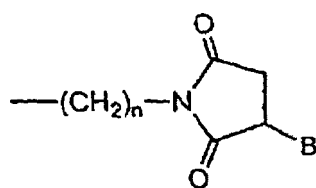


;

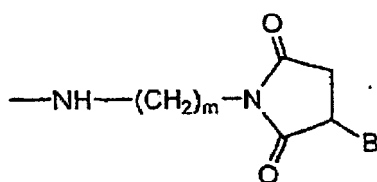


;

where Y'-B is

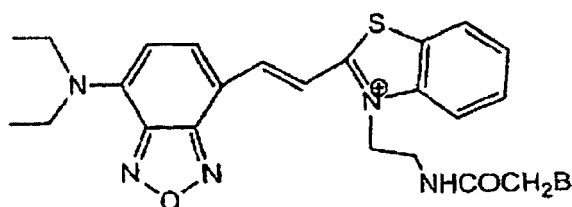


where n is an integer of 1 to 6,
or where $Y'-B$ is $A'-CO-V-B$, where A' is $-R^2O-$ or $-R^2N(R^3)-$, where R^2 is a C_1 to C_6 alkyl, R^3 is H or CH_3 , and $V-B$ is $-CH_2-B$ or

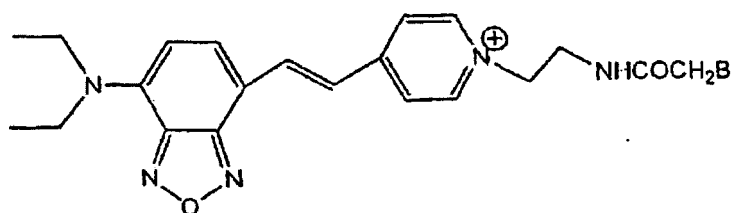


where m is an integer of 2 to 6,
where r is an integer of 1 to 3, R^3 and R^9 are independently a C_1 to C_6 alkyl or $(CH_2)_sCO_2H$, where s is an integer of 2 to 5, Z is S, O, or $C(CH_3)_2$, W is H, CH_3 , SO_3H , fused benzene, or fused sulfobenzene, and B is a receptor having a binding affinity for a ligand to be detected, and where said biosensor compound exhibits a detectable change in a fluorescence property as a result of changes in concentration of said ligand.

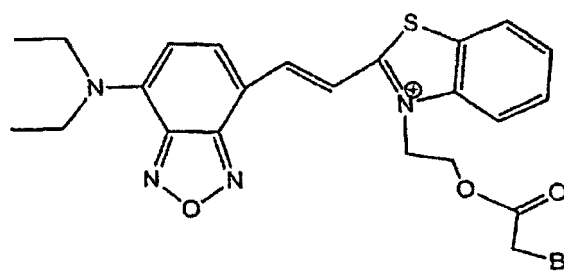
2. The biosensor compound of claim 1, wherein said biosensor compound is selected from the group consisting of



;



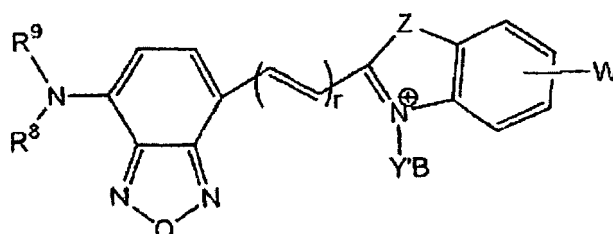
;

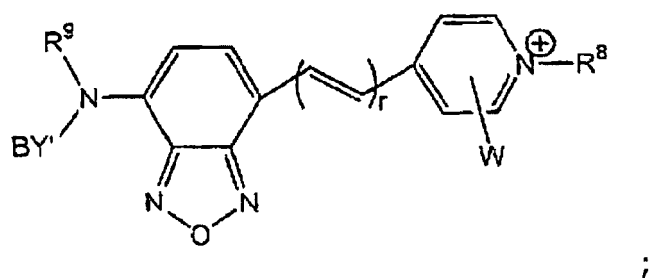
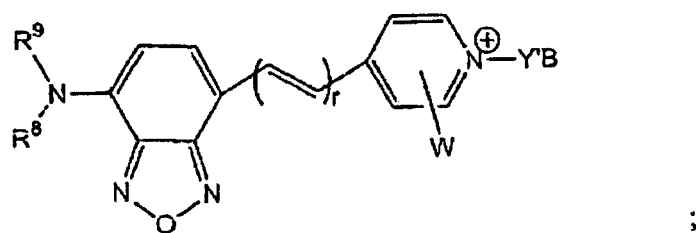
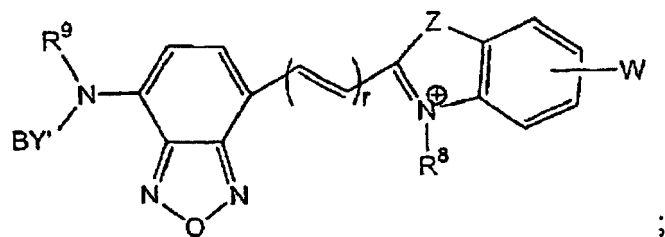


where B is as defined in claim 1.

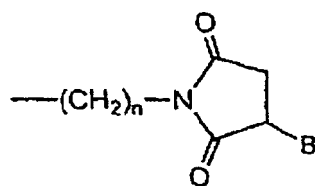
3. The biosensor compound of claim 1 or 2, wherein said receptor is a binding protein.
4. The biosensor compound of claim 1 or 2, wherein B is a periplasmic binding protein.
5. The biosensor compound of claim 1 or 2, wherein said biosensor fluorescence emission is above 575 nm.
6. The biosensor compound of claim 1 or 2, wherein B is a glucose/galactose binding protein (GGBP).
7. The biosensor of claim 3, wherein said binding protein is glucose/galactose binding protein having at least one amino acid substitution and where said at least one amino acid substitution is selected from the group consisting of a cysteine at position 11, a cysteine at position 14, a cysteine at position 19, a cysteine at position 43, a cysteine at position 74, a cysteine at position 107, a cysteine at position 110, a cysteine at position 112, a cysteine at position 113, a cysteine at position 137, a cysteine at position 149, a cysteine at position 213, a cysteine at position 216, a cysteine at position 238, a cysteine at position 287, a cysteine at position 292, a cysteine at position 112 and a serine at position 238, a cysteine at position 149 and a serine at position 238, a cysteine at position 152 and a serine at position 213, a cysteine at position 213 and a cysteine at position 238, a cysteine at position 149 and an arginine at position 213, a cysteine at position 149 and a serine at position 213 and a serine at position 238, and a cysteine at position 149 and an arginine at position 213 and a serine at position 238.
8. The biosensor compound of claim 1, wherein R^2O is $-CH_2CH_2O-$.
9. The biosensor compound of claim 1 wherein $R^2N(R^3)$ is $-CH_2CH_2NH-$.
10. A method for detecting analyte comprising:

providing a biosensor compound having at least one mutated binding protein with a fluorophore covalently attached thereto through a thiol group of said binding protein, where said fluorophore exhibits an emission fluorescence of at least 575 nm and is selected from the group consisting of

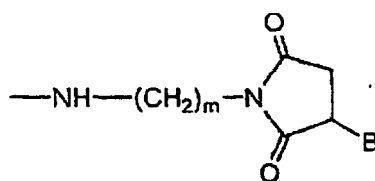




where Y'-B is

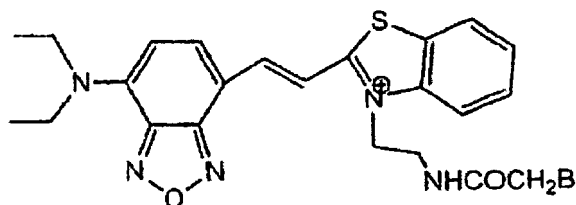


where n is an integer of 1 to 6,
or where Y'-B is A'-CO-V-B, where A' is -R²O- or -R²N(R³)-, where R² is
a C₁ to C₆ alkyl, R³ is H or CH₃, and V-B is -CH₂-B or

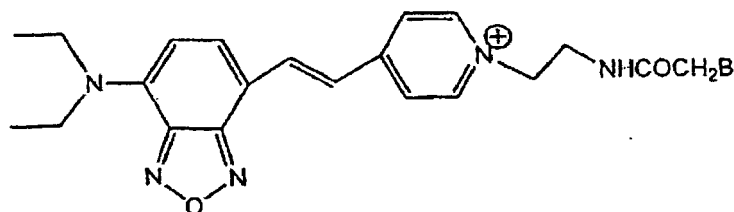


where m is an integer of 2 to 6,
 where r is an integer of 1 to 3, R⁸ and R⁹ are independently a C₁ to C₆ alkyl or (CH₂)_sCO₂H, where s is an integer of 2 to 5, Z is S, O, or C(CH₃)₂, W is H, CH₃, SO₃H, fused benzene, or fused sulfobenzene, and B is a receptor having a binding affinity for a ligand to be detected, and where said biosensor compound exhibits a detectable change in a fluorescence property as a result of changes in concentration of said ligand; contacting said binding protein with an analyte source to bind said analyte to said binding protein; and subjecting said binding protein to an energy source to excite said fluorophore and detecting a fluorescence property as an indicator of an analyte or analyte concentration in said analyte source.

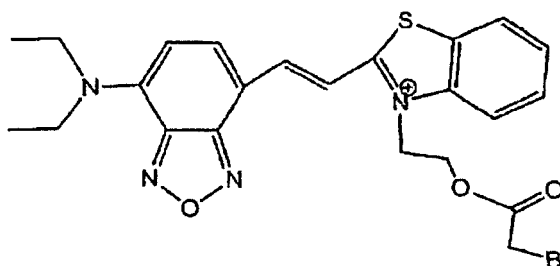
11. The method of claim 10, wherein said biosensor compound is selected from the group consisting of



;



;



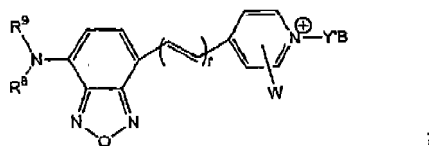
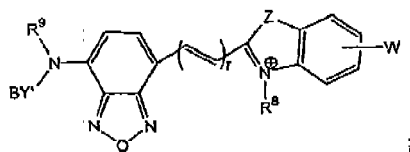
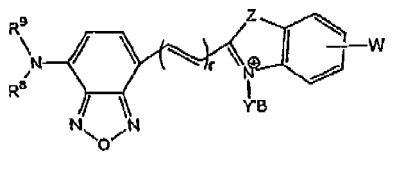
;

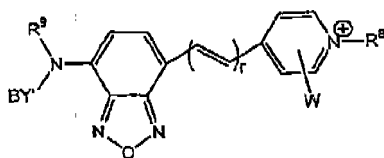
where B is as defined in claim 10.

12. The method of claim 10 or 11, wherein said binding protein is a periplasmic binding protein.
13. The method of claim 10 or 11, wherein said method comprises continuously contacting said binding protein with said analyte source, continuously subjecting said binding protein to said energy source and continuously detecting said fluorescence property.
14. The method of claim 10 or 11, wherein said mutated binding protein undergoes a conformational change as a result of changes in analyte concentration of said analyte source and where said method detects changes in said fluorescence property as a result of changes in said analyte concentration.
15. The method of claim 10 or 11, wherein said mutated binding protein is a glucose/galactose binding protein having at least one amino acid substitution selected from the group consisting of a cysteine at position 11, a cysteine at position 14, a cysteine at position 19, a cysteine at position 43, a cysteine at position 74, a cysteine at position 107, a cysteine at position 110, a cysteine at position 112, a cysteine at position 113, a cysteine at position 137, a cysteine at position 149, a cysteine at position 213, a cysteine at position 216, a cysteine at position 238, a cysteine at position 287, a cysteine at position 292, a cysteine at position 112 and a serine at position 238, a cysteine at position 149 and a serine at position 238, a cysteine at position 152 and a serine at position 213, a cysteine at position 213 and a cysteine at position 238, a cysteine at position 149 and an arginine at position 213, a cysteine at position 149 and a serine at position 213 and a serine at position 238, and a cysteine at position 149 and an arginine at position 213 and a serine at position 238.
16. The method of claim 10, wherein B is a glucose/galactose binding protein or a maltose binding protein.
17. The method of claim 10, wherein R^2O is $-CH_2CH_2-O-$.
18. The method of claim 10, wherein $R^2N(R^3)$ is $-CH_2CH_2NH-$.

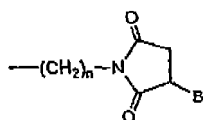
Patentansprüche

1. Biosensorverbindung, die aus der Gruppe ausgewählt ist, die aus Folgenden besteht:

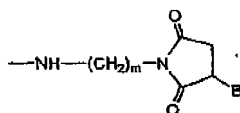




wobei Y'-B für

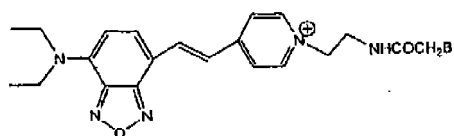
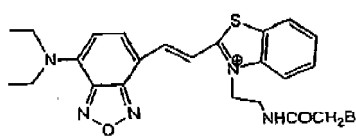


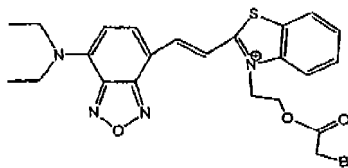
steht, wobei n eine ganze Zahl von 1 bis 6 ist,
oder wobei Y'-B für A'-CO-V-B steht, wobei A' = -R²O- oder -R²N(R³)- ist, wobei R² ein C₁- bis C₆-Alkyl ist, R³ = H
oder CH₃ ist und V-B = -CH₂-B oder



ist, wobei m eine ganze Zahl von 2 bis 6 ist;
wobei r eine ganze Zahl von 1 bis 3 ist, R⁸ und R⁹ unabhängig voneinander ein C₁- bis C₆-Alkyl oder (CH₂)₈CO₂H
sind, wobei s eine ganze Zahl von 2 bis 5 ist, Z = S, O oder C(CH₃)₂ ist, W = H, CH₃, SO₃H, anneliertes Benzol
oder anneliertes Sulfobenzol ist; und
B ein Rezeptor mit einer Bindungsaffinität zu einem nachzuweisenden Liganden ist und wobei die Biosensorver-
bindung als Ergebnis von Veränderungen der Konzentration des Liganden eine nachweisbare Veränderung in einer
Fluoreszenzeigenschaft zeigt.

2. Biosensorverbindung gemäß Anspruch 1, wobei die Biosensorverbindung aus der Gruppe ausgewählt ist, die aus

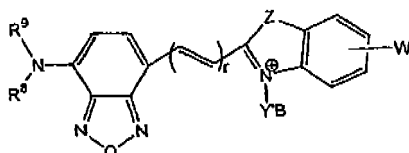


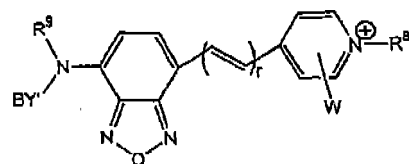
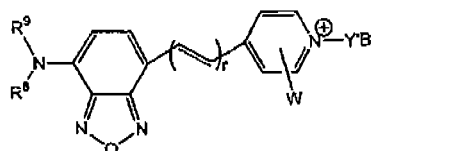
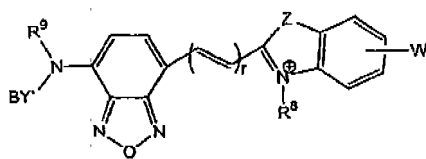


besteht, wobei B wie in Anspruch 1 definiert ist.

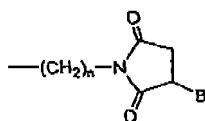
3. Biosensorverbindung gemäß Anspruch 1 oder 2, wobei der Rezeptor ein Bindungsprotein ist.
4. Biosensorverbindung gemäß Anspruch 1 oder 2, wobei B ein periplasmatisches Bindungsprotein ist.
5. Biosensorverbindung gemäß Anspruch 1 oder 2, wobei die Biosensor-Fluoreszenzemission oberhalb von 575 nm liegt.
6. Biosensorverbindung gemäß Anspruch 1 oder 2, wobei B ein Glucose/Galactose-bindendes Protein (GGBP) ist.
7. Biosensor gemäß Anspruch 3, wobei das Bindungsprotein ein Glucose/Galactose-bindendes Protein ist, das wenigstens eine Aminosäuresubstitution umfasst, und wobei die wenigstens eine Aminosäuresubstitution aus der Gruppe ausgewählt ist, die aus einem Cystein auf Position 11, einem Cystein auf Position 14, einem Cystein auf Position 19, einem Cystein auf Position 43, einem Cystein auf Position 74, einem Cystein auf Position 107, einem Cystein auf Position 110, einem Cystein auf Position 112, einem Cystein auf Position 113, einem Cystein auf Position 137, einem Cystein auf Position 149, einem Cystein auf Position 213, einem Cystein auf Position 216, einem Cystein auf Position 238, einem Cystein auf Position 287, einem Cystein auf Position 292, einem Cystein auf Position 112 und einem Serin auf Position 238, einem Cystein auf Position 149 und einem Serin auf Position 238, einem Cystein auf Position 152 und einem Serin auf Position 213, einem Cystein auf Position 213 und einem Cystein auf Position 238, einem Cystein auf Position 149 und einem Arginin auf Position 213, einem Cystein auf Position 149 und einem Serin auf Position 213 und einem Serin auf Position 238 sowie einem Cystein auf Position 149 und einem Arginin auf Position 213 und einem Serin auf Position 238 besteht.
8. Biosensorverbindung gemäß Anspruch 1, wobei $R^2O = -CH_2CH_2O-$ ist.
9. Biosensorverbindung gemäß Anspruch 1, wobei $R_2N(R^3) = -CH_2CH_2NH-$ ist.
10. Verfahren zum Nachweisen eines Analyten, umfassend:

Bereitstellen einer Biosensorverbindung, die wenigstens ein mutiertes Bindungsprotein aufweist, an das ein Fluorophor über eine Thiolgruppe des Bindungsproteins kovalent gebunden ist, wobei der Fluorophor eine Fluoreszenzemission von wenigstens 575 nm aufweist und aus der Gruppe ausgewählt ist, die aus Folgenden besteht:

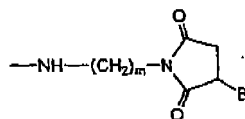




wobei Y'-B für

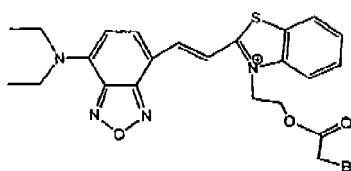
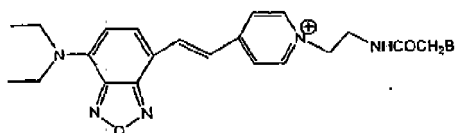
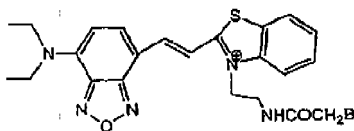


steht, wobei n eine ganze Zahl von 1 bis 6 ist,
oder wobei Y'-B für A'-CO-V-B steht, wobei A' = -R²O- oder -R²N(R³)- ist, wobei R² ein C₁- bis C₆-Alkyl ist, R³
= H oder CH₃ ist und V-B = -CH₂-B oder



ist, wobei m eine ganze Zahl von 2 bis 6 ist;
wobei r eine ganze Zahl von 1 bis 3 ist, R⁸ und R⁹ unabhängig voneinander ein C₁- bis C₆-Alkyl oder (CH₂)₅CO₂H
sind, wobei s eine ganze Zahl von 2 bis 5 ist, Z = S, O oder C(CH₃)₂ ist, W = H, CH₃, SO₃H, anneliertes Benzol
oder anneliertes Sulfobenzol ist; und
B ein Rezeptor mit einer Bindungsaffinität zu einem nachzuweisenden Liganden ist und wobei die Biosensor-
verbindung als Ergebnis von Veränderungen der Konzentration des Liganden eine nachweisbare Veränderung
in einer Fluoreszenzeigenschaft zeigt;
In-Kontakt-Bringen des Bindungsproteins mit einer Analytenquelle, um den Analyten an das Bindungsprotein
zu binden; und
Einwirkenlassen einer Energiequelle auf das Bindungsprotein, so dass der Fluorophor angeregt wird, und Nach-
weisen einer Fluoreszenzeigenschaft als Indikator für einen Analyten oder eine Analytenkonzentration in der
Analytenquelle.

11. Verfahren gemäß Anspruch 10, wobei die Biosensorverbindung aus der Gruppe ausgewählt ist, die aus



besteht, wobei B wie in Anspruch 10 definiert ist.

12. Verfahren gemäß Anspruch 10 oder 11, wobei das Bindungsprotein ein periplasmatisches Bindungsprotein ist.

13. Verfahren gemäß Anspruch 10 oder 11, wobei das Verfahren das kontinuierliche In-Kontakt-Bringen des Bindungsproteins mit der Analytenquelle, das kontinuierliche Einwirkenlassen der Energiequelle auf das Bindungsprotein und das kontinuierliche Nachweisen der Fluoreszenzeigenschaft umfasst.

14. Verfahren gemäß Anspruch 10 oder 11, wobei das mutierte Bindungsprotein als Ergebnis von Veränderungen der Analytenkonzentration in der Analytenquelle eine Konformationsänderung erfährt und wobei das Verfahren Veränderungen der Fluoreszenzeigenschaft als Ergebnis von Veränderungen der Analytenkonzentration nachweist.

15. Verfahren gemäß Anspruch 10 oder 11, wobei das mutierte Bindungsprotein ein Glucose/Galactose-bindendes Protein ist, das wenigstens eine Aminosäuresubstitution umfasst, und wobei die wenigstens eine Aminosäuresubstitution aus der Gruppe ausgewählt ist, die aus einem Cystein auf Position 11, einem Cystein auf Position 14, einem Cystein auf Position 19, einem Cystein auf Position 43, einem Cystein auf Position 74, einem Cystein auf Position 107, einem Cystein auf Position 110, einem Cystein auf Position 112, einem Cystein auf Position 113, einem Cystein auf Position 137, einem Cystein auf Position 149, einem Cystein auf Position 213, einem Cystein auf Position 216, einem Cystein auf Position 238, einem Cystein auf Position 287, einem Cystein auf Position 292, einem Cystein auf Position 112 und einem Serin auf Position 238, einem Cystein auf Position 149 und einem Serin auf Position 238, einem Cystein auf Position 152 und einem Serin auf Position 213, einem Cystein auf Position 213 und einem Cystein auf Position 238, einem Cystein auf Position 149 und einem Arginin auf Position 213, einem Cystein auf Position 149 und einem Serin auf Position 213 und einem Serin auf Position 238 sowie einem Cystein auf Position 149 und einem Arginin auf Position 213 und einem Serin auf Position 238 besteht.

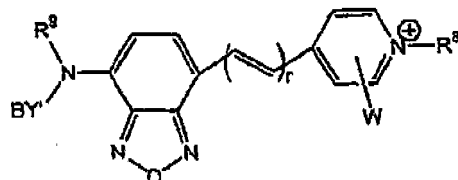
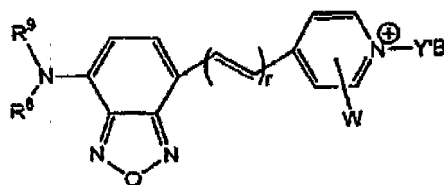
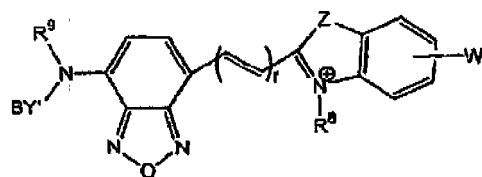
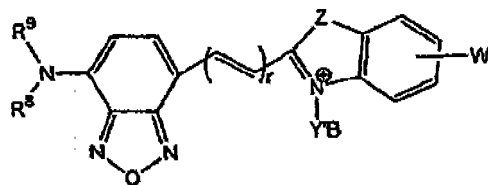
16. Verfahren gemäß Anspruch 10, wobei B ein Glucose/Galactose-bindendes Protein oder ein Maltose-bindendes Protein ist.

17. Verfahren gemäß Anspruch 10, wobei $R^2O = -CH_2CH_2-O-$ ist.

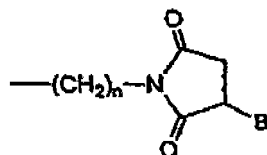
18. Verfahren gemäß Anspruch 10, wobei $R^2N(R^3) = -CH_2CH_2NH-$ ist.

Revendications

1. Composé biocapteur choisi dans le groupe constitué de :

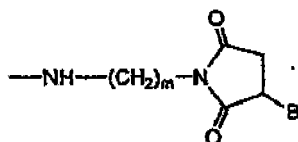


où Y'-B est



où n est un entier de 1 à 6,

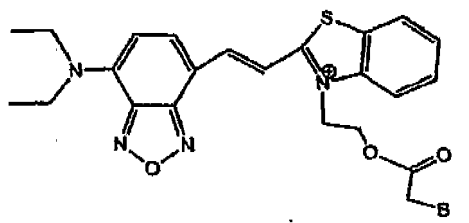
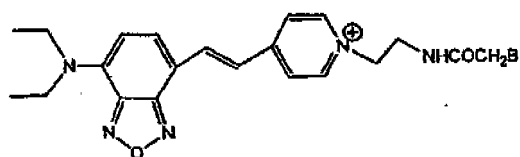
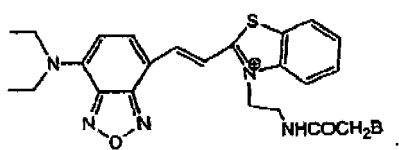
ou où Y'-B est A'-CO-V-B, où A' est $-R^2O-$ ou $-R^2N(R^3)-$, où R^2 est un alkyle en C_1 à C_6 . R^3 est H ou CH_3 , et V-B est $-CH_2-B$ ou



où m est un entier de 2 à 6,

où r est un entier de 1 à 3, R⁸ et R⁹ sont indépendamment un alkyle en C₁ à C₆ ou (CH₂)_sCO₂H, où s est un entier de 2 à 5, Z est S, O, ou C(CH₃)₂, W est H, CH₃, SO₃H ; un benzène fusionné, ou un sulfobenzène fusionné, et B est un récepteur ayant une affinité de liaison pour un ligand à détecter, et où ledit composé biocapteur présente un changement détectable d'une propriété de fluorescence en conséquence de changements de concentration dudit ligand.

2. Composé biocapteur de la revendication 1, où ledit composé biocapteur est choisi dans le groupe constitué de



où B est tel que défini dans la revendication 1.

3. Composé biocapteur de la revendication 1 ou 2, où ledit récepteur est une protéine de liaison.
4. Composé biocapteur de la revendication 1 ou 2, où B est une protéine de liaison périplasmique.
5. Composé biocapteur de la revendication 1 ou 2, où ladite émission de fluorescence de biocapteur est au-dessus de 575 nm.
6. Composé biocapteur de la revendication 1 ou 2, où B est une protéine de liaison de glucose/galactose (GGBP).
7. Biocapteur de la revendication 3, dans lequel ladite protéine de liaison est une protéine de liaison de glucose/galactose ayant au moins une substitution d'acide aminé et où ladite au moins une substitution d'acide aminé est choisie dans le groupe constitué d'une cystéine à la position 11, une cystéine à la position 14, une cystéine à la position 19, une cystéine à la position 43, une cystéine à la position 74, une cystéine à la position 107, une cystéine à la position 110, une cystéine à la position 112, une cystéine à la position 113, une cystéine à la position 137, une cystéine à la position 149, une cystéine à la position 213, une cystéine à la position 216, une cystéine à la position

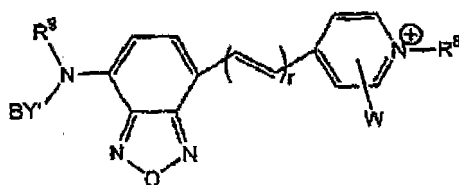
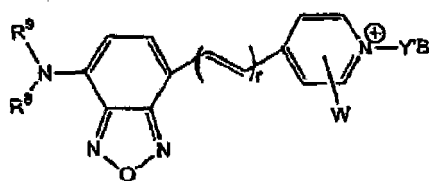
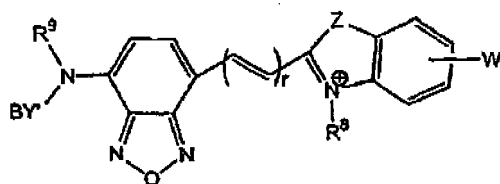
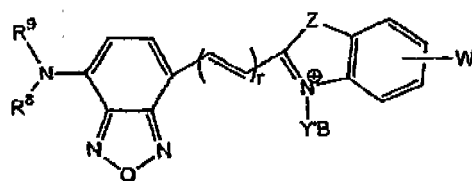
238, une cystéine à la position 287, une cystéine à la position 292, une cystéine à la position 112 et une sérine à la position 238, une cystéine à la position 149 et une sérine à la position 238, une cystéine à la position 152 et une sérine à la position 213, une cystéine à la position 213 et une cystéine à la position 238, une cystéine à la position 149 et une arginine à la position 213, une cystéine à la position 149 et une sérine à la position 213 et une sérine à la position 238, et une cystéine à la position 149 et une arginine à la position 213 et une sérine à la position 238.

8. Composé biocapteur de la revendication 1, dans lequel R^2O est $-CH_2CH_2O-$.

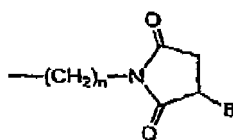
9. Composé biocapteur de la revendication 1 dans lequel $R^2N(R^3)$ est $-CH_2CH_2NH-$.

10. Procédé pour détecter un analyte comprenant :

la fourniture d'un composé biocapteur ayant au moins une protéine de liaison mutée avec un fluorophore lié de façon covalente à celle-ci par l'intermédiaire d'un groupe thiol de ladite protéine de liaison, où ledit fluorophore présente une fluorescence d'émission à au moins 575 nm et est choisi dans le groupe constitué de

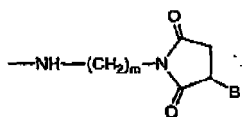


où $Y'-B$ est



où n est un entier de 1 à 6,

ou où Y'-B est A'-CO-V-B, où A' est -R²O- ou -R²N(R³)-, où R² est un alkyle en C₁ à C₆, R³ est H ou CH₃, et V-B est -CH₂-B ou



où m est un entier de 2 à 6,

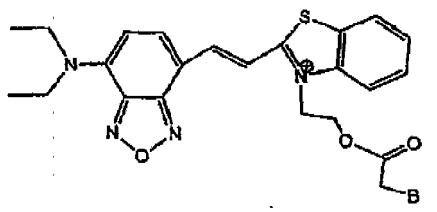
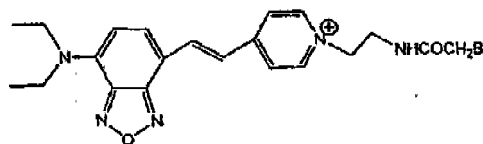
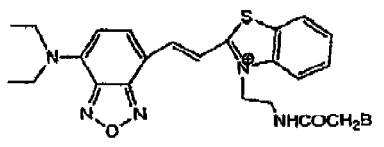
où r est un entier de 1 à 3, R⁸ et R⁹ sont indépendamment un alkyle en C₁ à C₆ ou (CH₂)_sCO₂H, où s est un entier de 2 à 5, Z est S, O, ou C(CH₃)₂, W est H, CH₃, SO₃H, un benzène condensé, ou un sulfobenzène condensé, et

B est un récepteur ayant une affinité de liaison pour un ligand à détecter, et où ledit composé biocapteur présente un changement détectable d'une propriété de fluorescence en conséquence de changements de concentration dudit ligand ;

la mise en contact de ladite protéine de liaison avec une source d'analyte pour lier ledit analyte à ladite protéine de liaison ; et

la soumission de ladite protéine de liaison à une source d'énergie pour exciter ledit fluorophore et détecter une propriété de fluorescence en tant qu'indicateur d'analyte ou de concentration d'analyte dans ladite source d'analyte.

11. Procédé de la revendication 10, dans lequel ledit composé biocapteur est choisi dans le groupe constitué de



où B est tel que défini dans la revendication 10.

12. Procédé de la revendication 10 ou 11, dans lequel ladite protéine de liaison est une protéine de liaison périplasmique.

13. Procédé de la revendication 10 ou 11, dans lequel ledit procédé comprend la mise en contact en continu de ladite protéine de liaison avec ladite source d'analyte, la soumission en continu de ladite protéine de liaison à ladite source d'énergie et la détection en continu de ladite propriété de fluorescence.

14. Procédé de la revendication 10 ou 11, dans lequel ladite protéine de liaison mutée subit un changement de confor-

mation en conséquence de changements de concentration d'analyte de ladite source d'analyte et où ledit procédé détecte les changements de ladite propriété de fluorescence en conséquence de changements de ladite concentration d'analyte.

- 5 **15.** Procédé de la revendication 10 ou 11, dans lequel ladite protéine de liaison mutée est une protéine de liaison de glucose/galactose ayant au moins une substitution d'acide aminé choisie dans le groupe constitué d'une cystéine à la position 11, une cystéine à la position 14, une cystéine à la position 19, une cystéine à la position 43, une cystéine à la position 74, une cystéine à la position 107, une cystéine à la position 110, une cystéine à la position 112, une cystéine à la position 113, une cystéine à la position 137, une cystéine à la position 149, une cystéine à la position 213, une cystéine à la position 216, une cystéine à la position 238, une cystéine à la position 287, une cystéine à la position 292, une cystéine à la position 112 et une sérine à la position 238, une cystéine à la position 149 et une sérine à la position 238, une cystéine à la position 152 et une sérine à la position 213, une cystéine à la position 213 et une cystéine à la position 238, une cystéine à la position 149 et une arginine à la position 213, une cystéine à la position 149 et une sérine à la position 213 et une sérine à la position 238, et une cystéine à la position 149 et une arginine à la position 213 et une sérine à la position 238.
- 10
- 15
- 16.** Procédé de la revendication 10, dans lequel B est une protéine de liaison de glucose/galactose ou une protéine de liaison de maltose.
- 20 **17.** Procédé de la revendication 10, dans lequel R^2O est $-CH_2CH_2-O-$.
- 18.** Procédé de la revendication 10, dans lequel $R^2N(R^3)$ est $-CH_2CH_2NH-$.

25

30

35

40

45

50

55